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Fluorescence Measurement of 805 nm Laser-Induced Release of 5,6-CF From DSPC Liposomes for Real-Time Monitoring of Temperature: An In Vivo Study in Rat Liver Using Indocyanine Green Potentiation

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Background and Objective: **This in vivo study examines the validity of using fluorescence measurements of laser-induced release of temperature-sensitive, liposome-encapsulated dye for real-time monitoring of temperature and for prediction of tissue thermal damage.**

Study Design/Materials and Methods: **An in vivo study is performed in rat liver after i.v. injection of liposomes loaded with a fluorescent dye and i.v. injection of indocyanine green (ICG) for diode laser potentiation. Temperature-sensitive liposomes (DSPC: Di-Stearoyl-Phosphatidyl-Choline) are loaded with 5,6-carboxyfluorescein (5,6-CF). These liposomes (1.5 ml solution) and ICG (1.5 ml solution-5mg/kg) are injected in adult male wistar rats. Two hours later, the liver is exposed and irradiated with a 0.8 W diode laser using pulses lasting from 1–6s (fluence ranging from 16–98 J/cm^2). Simultaneously, the fluorescence emission is analysed with an ultrahigh sensitivity intensified camera.**

Results: **The fluorescence intensity I_F increases linearly from 18 J/cm^2 up to 75 J/cm^2. These fluences correspond to surface temperatures between 42°C and 65°C. The measurements appear to be highly reproducible. In this temperature range, the accuracy is +/− 3°C. The maximum intensity is observed immediately after the laser is switched off. A decrease of the fluorescence intensity (27% in 20 minutes) is observed due to the 5,6-CF clearance. However, the ratio I_F/I_{BCK} (I_{BCK}: background fluorescence intensity) remains almost stable over this period of time and the determination of the temperature is still possible with good accuracy even 20 minutes after laser irradiation.**

Conclusion: **Real-time temperature monitoring by using fluorescence measurement of laser-induced release of liposome-encapsulated dye is clearly demonstrated. This procedure could conceivably prove useful for controlling the thermal coagulation of biological tissues.** © 1996 Wiley-Liss, Inc.

Key words: time-temperature history, temperature-sensitive liposomes, 805 nm diode laser, liver, photocoagulation, indocyanine green, 5-6-carboxy-fluorescein

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INTRODUCTION

We have already demonstrated the feasibility of using laser-induced release of liposome-encapsulated dye to monitor tissue temperature [1]. In that previous study, Nd-YAG irradiation was carried out in vivo in rat liver, and fluorescence measurements were performed ex vivo after the liver had been isolated and flushed with a saline solution.

The present in vivo study was undertaken to evaluate the sensitivity of fluorescence measurements to real-time temperature variations by monitoring the laser-induced release of a temperature-sensitive liposome encapsulated dye. The stability of the fluorescence intensity was also examined over a period of 20 minutes corresponding to the duration of the experimental procedure.

MATERIALS AND METHODS

Preparation of the Dye

56-carboxyfluorescein (Eastman Kodak) was used. The main excitation peak of this dye is 490 nm. The fluorescence emission is maximum at 515 nm. This carboxyfluorescein was purified using a procedure described by Ralston et al. [2]. This purification was achieved by treating with activated charcoal, washing, and finally performing hydrophobic chromatography on an Sephadex LH-20 (Pharmacia-Biotech, Sweden) column. Fractions were analysed by HPLC (HPLC: high pressure liquid chromatography) and purified fractions were pooled together and dessicated. A 100 mM solution was prepared by dissolving a suitable amount of the purified dye in water.

Preparation of Thermosensitive Liposomes

Temperature-sensitive liposomes used in this experiment were multilamellar vesicles. They were prepared by sonication, a procedure that has been described in a previous work [3]. A suitable amount of L-alpha-distearoyl phosphatidylcholine (DSPC: 18 carbon chains: liquid-crystal line phase transition temperature = 54°C, Sigma Chemical, St. Louis, MO) was dissolved in chloroform (Merck, Darmstadt, Germany). The phospholipid organic solution was evaporated under reduced pressure in a rotary evaporation flask. After complete removal of chloroform, a 100 mM 5,6-CF solution was added and hydratation of lipids was carried out at 55°C for 1 hour. After an equilibration period, the lipid suspension was sonicated at 55°C during 20 minutes (Sonicator Heat-System, Sonics & Materials, Danbury, CT) (500W, 10% output, 20,000 Hz, 3 mm diameter probe) under nitrogen. The liposome suspension was centrifuged at 4,000 rpm for 20 minutes (Heraus, Hanau, Germany) in order to eliminate titanium particles. Liposomes size was determined by quasi-elastic light scattering at a 90° angle (Sematech, SM 633/RTG, Nice, France). Mean size was determined to be 300 nm. The supernatant was dialyzed over 5 mM phophate-buffered saline (pH : 7.4) for 24 hours (1,000 ml, changed twice) to remove unencapsulated 5,6-CF. Immediately after dialysis, liposomes were injected to animals to prevent any leakage.

Animals

In vivo experimens were conducted on the livers of male wistar rats. The liver has many advantages as an experimental tissue in this study: (1) tissue characteristics are well known in the field of laser-induced thermal injury, (2) liposomes are cleared by the reticulendothelial system and are then concentrated in the liver, and (3) liposomes are homogeneously distributed in the liver due to liposome penetration of the liver during endocytosis in the liver cells (mainly Kupffer cells). The kinetics of liposome clearance from blood by liver show that liposome uptake by rat liver cells reaches a maximum 2 hours after liposome injection. Thus at the time of thermal injury, the liposomes are probably either adsorbed on the surface of hepatic cells or trapped in the intracellular compartments. The study was performed on five adult wistar rats weighing 400–600 g. The rats were anaesthetised and a solution of liposomes (1.5 ml) containing 5,6-CF was injected to each via the penile vein. Two minutes later, a solution (1.5 ml) of indocyanine green dye was injected via the penile vein. Two hours after the injections, a laparotomy was performed and the three main lobes of liver were exposed in order to perform laser irradiation and to record the fluorescence simultaneously.

Laser

It has already been demonstrated that the absorption of the laser light by the dye loaded in the liposome plays an important role in the heating that leads to dye release [4], making the choice of a wavelength crucial. The absorption of 5,6-CF is maximum at 0.49 μm, but there is no absorption of 5,6-CF in the red and infrared. For this reason, we chose an 805 nm diode laser (SDL 2372-P3). The smaller size and increased porta-

bility of the diode laser unit provided a convenient alternative to Nd:YAG laser emitting at 1.06 μm. The laser beam was transmitted throught a 400-μm optical fiber. Maximum power at the distal end of the fiber was 0.8 W. During the experiments, the distance between tissue and the fiber was kept constant (2 cm) with a mechanical holder to yield a 2.5-mm diameter spot. The laser beam profile and the beam diameter were controlled with a Beamscan (Photon).

Indocyanine Green Dye (ICG)

Due to the low power of the diode (0.8 W), we increased the 805 nm light absorption in rat liver with an injection of ICG, which is an indicator dye used for assessing cardiac output and liver function. It is also used for the examination of the retinal vasculature by fluorescence angiography. The absorption peak of ICG in plasma or blood is ~805 nm. After intravenous injection, ICG is bound to plasma proteins, mainly, alpha-1-lipoproteins, beta-1-lipoproteins, and albumin. ICG half-life is 3.4±0.7 minutes in blood. Following i.v. injection, ICG is rapidly excreted into bile with <4% of the total initial bolus dose remaining in the plasma after 20 minutes. However, hepatic ICG concentration increases with time to reach a maximum 2–4 hours following i.v. injection, and ICG may still be detected in the liver >12 hours after injection. Since Greenwell [5] showed that an ICG dosage of 5 mg/kg was capable of producing significant potentiation of 805 nm diode laser-tissue interaction in rat liver, we decided to use this dose. ICG (Serb, France) was reconstituted with sterile water immediately prior use. A volume of 1.5 ml (5 mg/kg) was injected via the penile vein 2 minutes after the liposome injection.

Fluorescence Imaging System

Fluorescence measurements were performed with a fluorescence imaging system developed in our laboratory. This system already has been described [6]. Briefly, fluorescence excitation was performed with a filtered Xenon lamp (490 nm interference filter, full width half maximum: FWHM = 10 nm). Fluorescence was analysed with an ultrahigh sensitivity intensified camera (model C2400-20, Hamamatsu Photonics, Hamamatsu City, Japan) composed of a low-noise, two-stage microchannel plate intensifier connected to a saticon tube. This camera was connected to an image processor (model Argus 50, Hamamatsu Photonics). Fluorescence images (512×512) were obtained every 40 ms on an image field of 40 mm × 40 mm (1,600 mm^2) at a distance of 35 cm from the liver. Since 5,6-CF was used, the peak fluorescence emission was selected using a narrow band interference filter (515 nn, FWHM = 1 nm, Corion, Holliston, MA). The amplification of fluorescence was calibrated to avoid any image intensifier saturation in case of intense fluorescence. Fluorescence intensities were displayed numerically (arbitrary units) by profiles or by 3D presentation.

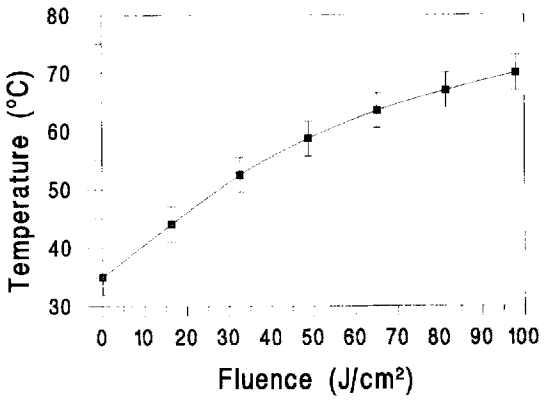

Fig. 1. Liver surface temperature increase as a function of fluence (805 nm diode laser, P = 0.8 W, Ø = 2.5 mm, t = 1 to 6 s, F = 16 to 98 J/cm^2) after previous i.v. injection of ICG (5 mg/kg) 2 hours before laser irradiation.

METHODS

This study consisted of using the predetermined diode-laser parameters (for a 5 mg/kg ICG dose) to obtain a temperature increase from the basal temperature up to 75°C. Since it was not possible to measure simultaneously the surface temperature and the fluorescence, a calibration curve linking the diode laser parameters for a 5 mg/kg ICG dose and the liver surface temperature was constructed. A preliminary phase of this experiment (data not shown) consisted of studying the relationship between the diode laser parameter following ICG injection and the liver surface temperature for a 5 mg/kg dose (Fig. 1).

In this in vivo study, fluorescence was measured immediately 5 minutes, 10 minutes, 15 minutes, and 20 minutes after the laser irradiation. The stability of the fluorescence intensity (I_F) of each spot was examined. The fluorescence measured in nonirradiated liver tissue was also studied (background fluorescence (I_{BCK})) and the ratio (I_F/I_{BCK}) was calculated. The phase transition temperature of the liposome was determined.

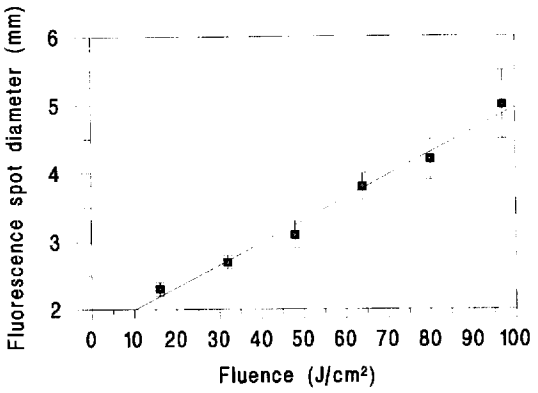

Fig. 2. Fluorescence spot diameter (mm) as a function of fluence (805 nm diode laser, P = 0.8 W, Ø = 2.5 mm, t = 1 to 6 s, F = 16 to 98 J/cm^2) after previous i.v. injection of ICG (5 mg/kg) 2 hours before laser irradiation.

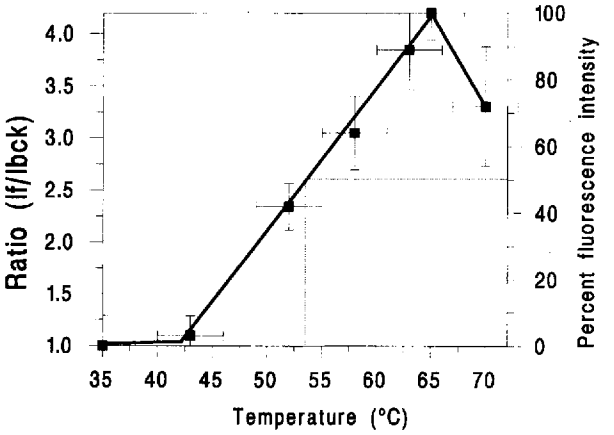

Fig. 3. Fluorescence intensity (%) and ratio (I_F/I_{BCK}) as a function of maximum surface temperature (805 nm diode laser, P = 0.8 W, Ø = 2.5 mm, t = 1 to 6 s, F = 16 to 98 J/cm^2, ICG = 5 mg/kg).

RESULTS

Fluorescence intensities were recorded at fluences ranging between 16 and 98 J/cm^2 (power = 0.8 W, spot diameter = 2.5 mm and pulse exposure time lasting from 1–6 s), corresponding to a maximum surface temperature between 35°C and 70°C. The irradiance was 16 W/cm^2. Between 20 and 25 laser-generated spots were analysed on each liver. However, for long duration pulses we sometimes observed elliptic spots instead of circular spots. These elliptic spots were mainly due to motions during rat breathing. Since the fluence was modified, these spots were not studied. Consequently, only 78 spots were analyzed.

We observed an enlargment of the fluorescence spots with increasing fluence (Fig. 2). The following equation gives the relation between the fluorescence spot diameter Ø (mm) and the fluence (J/cm^2):

$$Ø\ (mm) = 1.66 + 0.033\ (fluence)\ (r^2 = 0.98).$$

Figure 3 shows the percent fluorescence intensities measured at the center of the laser spot versus maximum temperature obtained at the same position using the calibration curve. Figure 3 also displays the ratio I_F/I_{BCK} versus temperature. It can be noted that the fluorescence increases linearly from 42°C (±3°C) up to 65°C(±3°C); 50% of maximum fluorescence is obtained at 53°C±3°C. A fluorescence intensity decrease is observed for temperature >65°C (fluence>75 J/cm^2). However, for irradiances >75 J/cm^2, a modification of the liver surface was observed. A fourfold increase of the ratio (I_F/I_{BCK}) is observed between 42°C and 65°C. The following equation gives the relation between the ratio (I_F/I_{BCK}) and the temperature T (°C) in the linear range:

$$T\ (°C) = 33 + 8\ R(I_F/I_{BCK})\ (r^2 = 0.98).$$

Figure 4 shows the influence of the delay between the irradiation and the fluorescence measurement. A decrease of both I_F and I_{BCK} is observed between 0 and 20 minutes, from 2,903 down to 2,122 for I_F and from 960 down to 746 for I_{BCK}. However, the ratio I_F/I_{BCK} is only slightly affected over this period of time: from 3.02 down to 2.84, corresponding to a temperature decrease from 57°C to 55.6°C (Fig. 5).

DISCUSSION

Our results confirm the previous study performed with a similar experimental procedure [1]. In that study, the animals were sacrificed a few minutes after Nd:YAG laser irradiation; the livers were surgically removed and flushed with a cooled saline solution. In the present study, the fluorescence measurements were performed on the living animal and the irradiation was performed with a 805 nm diode laser after previous ICG injection to potentiate the absorption of light. Thanks to that infrared absorbing dye, this study indicates that a 16 W/cm^2 irradiance and a 16–65 J/cm^2 fluence are sufficient to induce 5,6-CF release from liposomes instead of a 800 W/cm^2 irra-

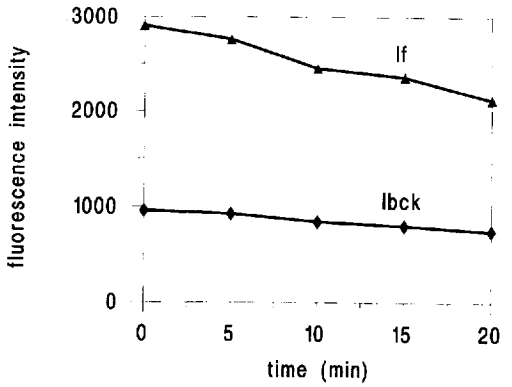

Fig. 4. Kinetic profiles of I_F and I_{BCK} as a function of time. (I_F was obtained after irradiation with the following parameters: 805 nm diode laser, P = 0.8 W, Ø = 2.5 mm, t = 3 s, F = 49 J/cm^2, ICG = 5 mg/kg.)

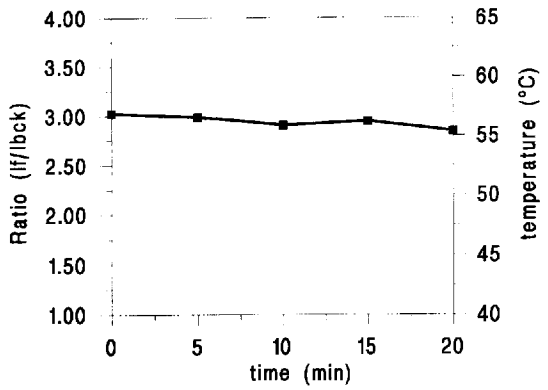

Fig. 5. Kinetic profile of ratio (I_F/I_{BCK}) as a function of time obtained from Figure 3. For T = 0 min, ratio (I_F/I_{BCK}) = 3.02 and θ = 57°C and for T = 20 min, ratio (I_F/I_{BCK}) = 2.84 and θ = 55.6°C.

diance and a 80–200 J/cm^2 fluence using a 1.06 μm Nd:YAG laser.

Figure 3 shows that fluorescence intensity plateaus for 4s and 5s pulses corresponding to fluences of 65 and 81 J/cm^2. For these fluences, one can consider that dye release from the liposomes is maximum. An increase of temperature causes, in fact, further lysing of surrounding liposomes because of the transfer of heat to the surrounding area. This was observed in our study (Figure 2) and by Khoobehi [7].

For 6s pulses, modification of the liver surface was observed. This modification acts as an "optical shield" that strongly absorbs the fluorescence light. Consequently, a fluorescence intensity decrease down to 70% is observed. The fluorescence intensity variations are also important. This phenomenom was also observed by Khoobehi in a different model (blood inside capillary tube). In our study, the fluorescence intensity increases linearly between 42°C±3°C and 65°C±3°C and confirms the previous study [1]. This appears to be well adapted to the control of the photocoagulation of biological tissues, since the critical coagulation temperature is usually considered to be 53°C for the liver and ~55°C for most biological tissues [8].

Approximatively 50% of the fluorescence intensity is obtained at 53°C. This corresponds exactly to our previous study [1]. The phase transition temperature of DSPC liposomes is considered to be 54°C±2°C [3]. Consequently, we can consider that the release is due only to the tissue temperature increase.

The ratio (I_F/I_{BCK}) increases as a function of temperature between 42°C and 65°C. Figure 3 shows a fourfold increase of the ratio (I_F/I_{BCK}) between 42°C and 65°C. This ratio remains almost stable over a period of 20 minutes corresponding to the duration of the experimental procedure (Fig. 5). The calculated temperature at 20 minutes is 55.6°C compared to 57°C immediately after irradiation (t = 0 min). This difference of 1.4°C is accounted for temperature by the measurement accuracy ±3°C.

If the ratio I_F/I_{BCK} is only slightly affected over this period of time, a fluorescence intensity decrease is observed for both irradiated and nonirradiated biological tissues. Over this 20-minute period, a 27% decrease of fluorescence intensity is observed (Fig. 4). This finding was expected for two reasons: (1) We know that although liposomes are dialyzed before injection, free 5,6-CF is present in tissue. In fact, when DSPC liposomes are injected i.v., destabilization of the phospholipid bilayer occurs by proteins binding and by phospholipids exchanging with the lipoproteins. This phenomenon induces a little release of the dye (a few %). The presence of free 5,6-CF leads to the appearance of the fluorescence background. (2) The hepatic clearance of 5,6-CF in liver is slower than this in the blood. We have studied the kinetic clearance of 5,6-CF in liver and measured a half-life of 40 minutes (data not shown) compared to a 5-minute half-life in blood. This explains why it is possible to measure the liver background fluorescence >1 hour after laser irradiation.

Consequently, in the case of liver it is possible to determine the peak temperature even after the laser irradiation. Conventional techniques allow the measurements of the temperature and the determination of the peak temperature only during the laser irradiation.

CONCLUSION

This study confirms our preliminary results obtained ex vivo. The fluorescence measurement of the laser-induced release of liposome-encapsulated dye allows a real-time temperature monitoring. Consequently, a real-time tissue damage control seems feasible. This procedure could conceivably prove useful for controlling the thermal coagulation of biological tissues such as blood vessels.

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REFERENCES

1. Mordon S, Desmettre T, Devoisselle JM. Laser-induced release of liposome-encapsulated dye to monitor tissue temperature: A preliminary in-vivo study. Lasers Surgery Med 1995; 16:246–252.
2. Ralston E, Hjelmeland LM, Klausner RD, Weinstein JN, Carboxyfluorescein as a probe for liposome-cell interactions: Effect of impurities and purification of the dye. Biochimica Biophysica Acta 1981; 649:133–137.
3. Magin RL, Niesman MR. Temperature-dependent drug release from large unilamellar liposome. Cancer Drug Delivery 1984; 1,2:109–117.
4. Khoobehi B, Char CA, Peyman GA, Schuele KM. Study of the mechanisms of laser-induced release of liposome-encapsulated dye. Lasers Surg Med 1990; 10:303–309.
5. Greenwell TJ, Wyman A, Rogers K. Potentiation of laser-tissue interactions of the 805 laser with indocyanine green. Lasers Med Sci 1993; 8:283–287.
6. Mordon S, Devoisselle JM, Maunoury V. In vivo fluorescence imaging of tumorous tissue using a pH-sensitive indicator (5,6-Carboxyfluorescein): Instrumental and experimental studies. Photochem Photobiol 1994; 60, 3:274–279.
7. Khoobehi B, Char CA, Peyman GA, Assessment of laser-induced release of drugs from liposomes: An in vitro study. Lasers Surg Med 1990; 10:60–65.
8. Welch AJ. Laser irradiation of tissue. In Shitzer A, Eberhart RC, eds. "Heat Transfer in Medicine and Biology," Vol. 2. New York: Plenum Press, 1985, pp 135–179.